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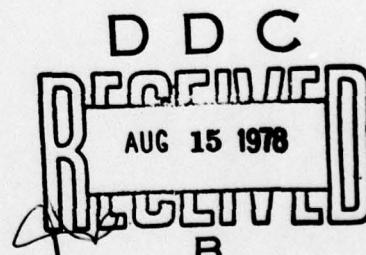
6 SYMPOSIUM ON FUNCTIONS OF MICROBIAL MEMBRANES  
TUBINGEN, GERMANY, 5-7 SEPTEMBER 1977

10 JOHN E. SIPPEL

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SYMPOSIUM ON FUNCTIONS OF MICROBIAL MEMBRANES  
TÜBINGEN, GERMANY, 5-7 SEPTEMBER 1977

INTRODUCTION

The Symposium on Functions of Microbial Membranes, which was held at the University of Tübingen on the occasion of its 500 years' anniversary, was sponsored by the University and the Deutsche Forschungsgemeinschaft. It was most appropriate that such a meeting be held at Tübingen since it was here that serious investigation of membranes began 25 years ago by Weidel, who studied phage absorption and membrane changes, and currently there is exceptional expertise here on membrane structure and function. There were twenty-four 40-minute formal papers and 48 poster presentations during the 3-day program. The 124 microbiologists who attended the meeting were from Europe, the United States, Australia, and Japan.

CELL ENVELOPES AND GRAM-NEGATIVE BACTERIA

Most of the material presented at the Symposium pertained to cell envelope structures in Gram-negative bacteria, a subject that was recently reviewed by Costerton *et al* (*Bact. Rev.* 38:87-110). A cell envelope is composed of a cytoplasmic membrane (CM) and a cell wall (CW). The former is a lipoprotein structure that produces trilamellar images on electronmicrographs. The hydrophobic zone of the CM is traversed by protein "studs" which may be permease molecules. Enzymes and binding proteins are associated with CM, and the basic chemical units of the CW are synthesized by CM enzymes. The CW consists of a rigid peptidoglycan layer that provides the cell's shape and an outer membrane (OM) that is structurally similar to the CM. Peptidoglycan of Gram-negative bacteria has a relatively open molecular structure and is therefore no barrier to small molecules. However, alterations to the peptidoglycan layer result in pronounced changes in penetrability of OM which suggests that peptidoglycan provides foundation for other CW components. Lipoproteins extend from the peptidoglycan layer to the OM: The lipid portion may actually be bound to the OM by hydrophobic interactions, thereby anchoring the OM to the peptidoglycan layer. The periplasmic zone is that which extends from the CM to the OM and is occupied by the peptidoglycan-lipoprotein complex and periplasmic spaces. If the OM is disrupted, periplasmic enzymes, binding proteins, and pigments are released. The enzymes are evenly distributed throughout the periplasmic zone, and available evidence suggests that they are bound to specific structural components. Binding proteins appear to be associated with the outside of the CM.

The OM of Gram-negative CW envelopes contain phospholipids, proteins, and Lipopolysaccharides (LPS). As with the CM, the basic structure is formed by proteins and phospholipids; the lipid A portion of the LPS is associated with the hydrophobic zone and the oligosaccharide portion

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with inner and outer surfaces of the OM. The phospholipids of the OM are quantitatively similar to, but qualitatively different from, those of the CM. The proteins also differ from those in the CM, and there is evidence that up to 70% of OM protein is contained in a few major protein species. There is apparently no active transport in the OM; the protein "studs" of the membrane may function as "molecular sieves" by providing transport for molecules under a certain size. LPS contributes to the barrier functions of the OM since there is a marked increase in inward penetration of antibiotics and release in periplasmic enzymes in rough *S. typhimurium*. An intact "picket fence" of polysaccharide chains (O antigens) also protects smooth strains by reacting with antibodies and complement at a distance from OM. The specificity at phage receptor sites is determined by the terminal sugar portion of LPS molecules; thus phage receptors are composed of both lipoprotein and LPS. Colicin receptors, on the other hand, are single exposed glycoproteins.

"Arrangement and Properties of *E. coli* Outer Membrane Proteins,"  
by U. Henning, Max-Planck-Institut für Biologie, Tübingen

Major OM proteins from *E. coli* have been labelled with different nomenclatures by different investigators. Henning provided an invaluable contribution to the Symposium at its outset by comparing these terminologies.

<u>MW(K)</u>	<u>Bragg</u>	<u>Henning</u>	<u>Lugtenberg</u>	<u>Mitsusshima</u>	<u>Rosenbusch</u>	<u>Schnaitman</u>
39			a	0-7	matrix	3b
	A1	Ia	b	0-9		la
37	A2	Ib	c	0-8	protein	lb
		Ic				
33	B	II*	d	0-10 0-11		2 3a
17		III				

(a - 0-7 3b is present only at higher growth temperatures; 2 is present only in phage PA-2 lysogens; 1c is a new, third variant of protein I)

There is abundant evidence that there are several species of protein I. Henning suggests that protein I is a temperature-sensitive protein, and different molecular weights (MW) result depending on degree of reformation after heat destruction. Comparable heterogeneity seems to exist for protein II\* (pronounced two star). Proteins Ia, Ib, and II\* serve as receptors for specific phages, and these receptor functions require the presence of LPS. Henning suggested that protein I also functions as a pore for carbohydrate transport, and protein II\* as a pore for amino acid transport.

"Defective Growth Functions of *ompA* Mutants of *Escherichia coli* K-12," (poster presentation), by P.A. Manning, University of Adelaide, Australia

Further evidence for protein II\* (3a) functioning in amino acid transport was given in this study. Mutants defective in this protein were shown to grow less well than parent strains under a variety of conditions and to be defective in uptake of amino acids. However, the uptakes of the larger substrates ferrienterochelin and cyanocobalamin (vitamin B12) were normal. A possible explanation for the reduced ability of *ompA* mutants to accumulate amino acids is that leakage is more rapid.

"Assembly of Outer Membrane Proteins on the Cell Surface of *E. coli*," by S. Mizushima, Nagoya University, Japan.

Major OM proteins O-8, O-9, and O-10 can be purified without heat in sodium dodecyl sulfate (SDS). The protein O-8 was more stable than O-9 in SDS, thereby suggesting that they are different. Since tertiary structure of all three can be maintained, it was possible to study their interactions with peptidoglycan or lipopolysaccharide. Only O-8 and O-9 combined completely with peptidoglycan. The extent of binding with purified O-8 or O-9 was significantly decreased with increased heat, NaCl concentration, or Mg concentration. Proteins O-8 and O-9 are very similar (terminal amino acids for O-8 are Ala-Glu-Val-Tyr-AspN; for O-9 they are Ala-Glu-Ile-Tyr-AspN) and probably interact with the same site on the peptidoglycan layer (diaminopimelic acid on the surface). Proteins O-8 and O-9 can also interact with LPS.

"Biogenesis of the Outer Membrane of *Salmonella*," by M. J. Osborn, University of Connecticut Health Center, Farmington, CT.

LPS and lipoproteins are synthesized in the CM and translocated over periplasmic spaces to the OM. OM phospholipids differ from those in the CM, and LPS is found almost exclusively in the OM. Translocation of LPS appears to be irreversible and to occur at specialized areas of contact between the two membranes (zones of adhesion). In order to study phospholipid translocation exogenous lipids were introduced into the OM of intact cells from small bilayer phospholipid vesicles. The process required calcium ions, was non-specific with respect to vesicle composition, but dependent on the nature of cellular LPS ( $R_e > R_c > S$ ). The rate at which the lipids were taken up from the vesicles was proportional to the vesicle concentration. Phospholipids incorporated into the membrane by this technique were translocated to the CM by a process which was rapid, reversible, and relatively non-specific with respect to phospholipid species. The results suggest diffusional flow of these lipids through zones of adhesion. In contrast, LPS remained in the OM after incorporation from mixed lipid-vesicles. It is suggested that irreversible capture of LPS arises from secondary LPS-protein interactions within the OM.

"Junctions between Inner and Outer Membrane. The Role of Cell Surface Structures During Infections by LPS-Specific and Capsule-Specific Phages," by M. E. Bayer Institute for Cancer Research, Philadelphia, PA.

Viruses, when infecting host cells, go through a series of events that start with virus-cell collision and end with release of the virus nucleic acid. Bayer divided the sequential events into four steps:

- 1) Collision may be followed by a two-dimensional random walk of the virus particle along the cell surface until it attaches to the LPS or capsule polysaccharide receptor, or it may detach and become available again for collision.
- 2) Penetration/degradation of the receptor polymers. The phages penetrate the domain occupied by the polysaccharide in less than a minute. Two types of interactions are involved: (a) Viruses breaking the glycosidic linkages cause a rapid destruction of the polymeric superstructure; a dislodging of larger pieces of capsular material results with desorption of infectious virus particle. (b) A virus with esterase activity does not have such an effect; most of the virus particles remain attached to the cell.
- 3) Orientation of the virus over the membrane adhesion sites. In over 80% of the cases, the virus particles are seen over the area of the adhesion site.
- 4) Release of virus nucleic acid. Correlated with this are changes in the adsorption apparatus and in the envelope of the cell.

The role of the adhesion site in these systems is twofold: (a) They are the sites of growth, with insertion of capsular antigens, LPS, F pili, and flagellae, which also serve as phage receptors, and (b) they generate the signal(s) for release of the virus nucleic acid. Excellent electron micrographs of these sites were presented which show bacterial chromosomal material to extend almost into the structure.

"Outer Membrane Transport and the Colicin E Receptor," by C. Schnaitman, University of Virginia Medical School, Charlottesville, VA.

The cell surface receptor for colicins of the E group is a 60,000 MW protein, of which there are approximately 200 copies per cell, and is the product of the bfe gene. This protein is also involved in vitamin B<sub>12</sub> transport, presumably by functioning as a binding site. A tonB gene product is required for B<sub>12</sub> transport but not for B<sub>12</sub> binding or colicin E action. The close linkage between bfe gene and the gene for arginine synthesizing enzymes allowed the study of the role of bfe gene product in colicin killing by construction of arg<sup>+</sup> F<sup>+</sup> strains carrying different bfe alleles, and mating these with arg strains. At various times after mating, arg<sup>+</sup> recombinants were treated with colicin E3 or phage BF23, and survivors were detected on arginine-free medium. Similar experiments on the functional stability of the bfe and tonB gene products were done by studying B<sub>12</sub> transport and colicin sensitivity in bfeAm and tonBAm mutants in a strain carrying a temperature-sensitive Am suppressor after shift in temperature. The results of these experiments indicate that the bfe gene product is stable with respect to its role in B<sub>12</sub> transport after it is inserted in the OM, but that the tonB gene product is labile and must be synthesized continuously for functional transport. On the other hand, only newly-synthesized bfe gene product can function in colicin killing, although "old" protein can still bind colicin. This indicates that the colicin receptor must sit over a sensitive site, possibly a fusion site between OM and CM (adhesion site) in order to mediate killing. The receptor can move away from such sites by membrane growth, but inhibition of protein synthesis with chloramphenicol appears to "freeze" the receptor in "place."

"The Structural Basis of the Selective Permeability Properties of the Outer Membrane," by H. Nikaido, University of California, Berkeley, CA.

OM serves primarily as a permeability barrier. The mechanisms by which various compounds overcome this barrier appear to be complex. At present at least four types of mechanisms can be distinguished:

	<u>Hydrophilic</u>	<u>Hydrophobic</u>	<u>Macromolecules</u>
	<u>Specific</u>	<u>Phospholipid Bilayer</u>	<u>Unstable OM Structures</u>
<u>"Porin"</u>	<u>Protein</u>		
Non-specific MW 600 Sugars Amino acids Nucleotides Small hydrophilic anti-biotids	Vitamin B <sub>12</sub> Ferricin Maltose Nucleoside	Novobiocin Rifampicin Crystal Violet	Lysozyme Periplasmic enzymes <i>(OM unstable, breaks and repairs constantly, allowing molecules to penetrate. Theory only)</i>

The permeability property of "porins" was reproduced *in vitro* in vesicle membranes reconstituted from isolated phospholipids, lipoproteins, and OM proteins by Nakai (Tokai University, Japan). The constituent that could confer such permeability to the reconstituted vesicles was the OM protein with molecular weights around 35,000 Daltons ("porins"). Each of the 34K, 35K, and 36K "porins" produced by *Salmonella typhimurium* produced transmembrane permeability channels in reconstituted vesicle membranes. The exclusion limits of the pores made of the "porins" were indistinguishable from each other.

The following table suggests that other factors besides "porins" may be involved in transport of small hydrophilic molecules:

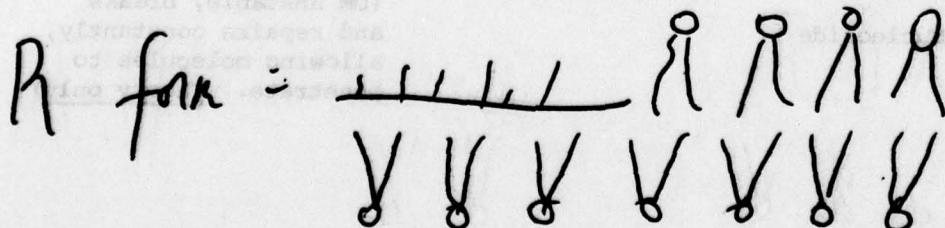
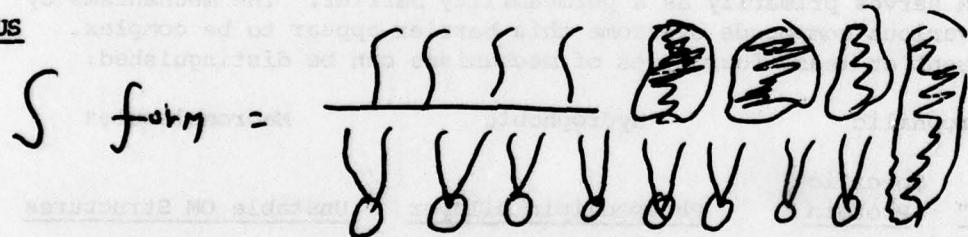
**OM Permeability Estimated from Transport "KM"**

"Porins"	$\lambda$ -Receptor	Permeability		
		Glu	Lac	His
+	-	>400	>100	>100
-	-	20	2	>30
-	+	50	20	>30

therefore -  $\lambda$  receptors contribute to glucose and lactose transport.

Hydrophobic molecules tend to be excluded from the pores. Nikaido has demonstrated that OM shows little permeability to many hydrophilic molecules that penetrate the OM of "deep rough" mutants. His studies suggest that the barrier property of the cited type membrane is related to the presence of few phospholipid molecules in the outer leaflet of the OM.

THUS



"Interaction of Cloacin and Cloacin Complex with the Outer and Inner Cell Membrane of Sensitized Cells," (Poster Presentation), by B. Oudega, Free University, Amsterdam

Cloacinogenic cells of a strain of *Enterobacter cloacae* produce a bacteriocin which is an equimolar complex of cloacin, which has MW of 56,000 Daltons, and its immunity protein, MW 10,000 Daltons. The complex is absorbed onto specific OM receptors, transferred towards the CM, and interacts with the CM resulting in leakage of potassium ions and finally inhibition of protein synthesis. The killing activity of the complex is strongly reduced by removal of the immunity protein. The absorption properties of cloacin and cloacin complex are very similar, and there is no significant difference in the affinities of both preparations for isolated CM vesicles. However, cloacin alone, when attached to reception sites, can be digested by trypsin for much longer periods of time than the cloacin complex. It is concluded that removal of immunity protein from the cloacin complex results in reduction of the transfer of active cloacin molecules from OM to CM.

"Synthesis of Cell Envelope Components by Anucleate Cells (Minicells) of *Bacillus subtilis*," (Poster Presentation), by G. Mertens, Max-Planck-Institut für Molecular Genetik, Berlin

Minicells are small anucleate cells that serve as excellent tools for examining gene products of plasmids and the DNA encoded by bacteriophages that infect the minicells. It has been reported that minicells without plasmids or phage DNA can produce OM protein from amino-acid precursors apparently because of the presence of stable messenger RNA. This study involved synthetic abilities in Gram-positive DNA-less cells, that is, those without OM. The minicells were produced by growing *B. subtilis* CU403 in minimal medium and separating minicells from nucleate cells by differential centrifugation. The minicells were shown to be able to synthesize mucopeptide by incorporation of L- and D-Alanine and N-acetylglucosamine into trichloroacetic acid-precipitable material which is solubilized by lysozyme digestion, and by inhibition of this incorporation by inhibition of CW synthesis. These observations demonstrate that cell envelope biosynthesis can occur in the absence of DNA and that the enzymes responsible are active for long periods *in vivo*.

"Subversion of Facilitated Transport Mechanisms by Antimicrobial Agents," by T. J. Franklin and G. A. Snow, Imperial Chemical Industries Limited, Cheshire, UK

A more applied approach to bacterial membrane function was provided in this report. Most antimicrobial agents penetrate bacterial cell envelopes by simple diffusion. However, some drugs utilize CM transport systems for this purpose. In some cases, there is a structural resemblance between the transported drug and a nutrient, e.g., cycloserine

and D-Alanine. High concentrations of these drugs can be obtained in bacterial cells by this process. The facilitated transport of the tetracyclines is less easy to explain but may depend on an affinity for a divalent cation transport system. The oligopeptide permease system, which is not essential for bacterial survival, has also been investigated as a mechanism for drug uptake. Some tripeptides are toxic to bacteria; tri-ornithine is quite toxic, but there is a high frequency of resistance. However, a lower frequency of resistance was obtained with Gyl-Gly-Val. Other combinations are being investigated. Sulphonilic acid is not an effective antimicrobial by itself because it does not penetrate. The authors report some success with sulphonilic acid when it is complexed with Phe-Gly. However, strains appear to differ in their capacity to absorb it.

"Biogenesis of the Purple Membrane of Halobacteria," by M. Sumper, Institut für Biochemie der Universität Würzburg

The purple membrane of these organisms makes an excellent model system for the study of membrane biogenesis. Its synthesis can be induced by lowering the oxygen tension or inhibited with nicotine. Its chemical composition is simple. Bacterio-opsin (MW 26,000) is the only membrane protein present. Unlike the lipid components of the purple membrane, membrane protein and pigment synthesis are strictly controlled. The biological function of purple membrane is to produce energy from light resulting in ATP production.

"Cell-Cell Interactions in Bacterial Conjugation," by M. Achtmann, Max-Planck-Institut für molekulare Genetik, Berlin

Initial cell-cell interactions involve F pilus contacts, unstable wall-to-wall contacts, and finally stable wall-to-wall contacts. Once stable wall-to-wall contacts are formed, DNA transfer occurs, even after removing F pili (with SDS). The function of the F pilus is not entirely clear, although it is apparently required for cell contact. The F factor makes a cell a donor and prevents it from being a receptor ( $F^+$  to  $F^+$  interactions occur with a frequency 1/200th that of  $F^+$  to  $F^-$ ). Seventy percent of F protein is OM. Cells in a mating mixture that are involved in conjugation are found in aggregates containing up to 50 cells; sometimes there will be one donor and many recipients or one recipient with many donors. DNA transfer occurs with 100% efficiency per contact. Termination of DNA transfer is rapidly followed by active disaggregation.

F pili have been purified and found to contain a single protein subunit, and they can bind specifically to *E. coli* cell surfaces. Purified protein II\* has been shown to be involved in the stabilization of wall-to-wall contacts; it is not the F pilus receptor.

**ENTEROBACTERIAL COMMON ANTIGEN (ECA)** (For a review of ECA see Makela and Mayor, *Bact. Rev.* 40:591-632).

All strains of the family Enterobacteriaceae (with the exception of defective mutants) share an antigen component, the ECA. ECA is not present in other Gram-negative bacteria nor in Gram-positive bacteria. The antigen is demonstrable by indirect hemagglutination; however, although all strains have the antigen, most do not produce anti-ECA antibodies. ECA is generally present in a "haptenic form" which has a propensity to aggregate with hydrophobic structures like LPS. In some organisms, ECA is linked to LPS.

Chemical characterization of "purified" ECA suggests that the main component contains a heteropolymer of D-glucosamine and D-mannosaminuronic acid. The latter sugar appears essential for the serological specificity of ECA. Some fatty acid, mainly palmitic, is linked to the polymer. ECA therefore seems to be an amphiphilic molecule in which the sugar units are responsible for immunological specificity and the hydrophobic part for an aggregation tendency. The LPS-linked form of ECA (the immunogenic form) has the same immunological specificity and therefore should contain mannosaminuronic acid. Mutants blocked in ECA biosynthesis have been found that indicate that there is an interrelationship between biosynthesis of ECA and LPS.

Since anti-ECA antibodies are found in high titers in patients infected with enteric bacteria, these antigens have potential in diagnosis and prophylaxis of such diseases. There were two poster presentations on ECA at the Symposium.

"Enterobacterial Common Antigen - I. Studies on the Haptenic Form," by D. Mannel, Max-Planck-Institut für Immunbiologie, Freiburg i/Breisgau

The haptenic form of ECA was isolated from *Salmonella montevideo*. The phenol-soluble material was purified by diethylaminoethylcellulose chromatography. Two precipitation bands were found on immunoelectrophoresis when this ECA was tested against a high-titered antiserum that had been absorbed with a homologous ECA-negative mutant. No differences were found in the composition of these two antigenic species. A comparison of the ECA-content of *Proteus mirabilis* L-forms, spheroplasts, and normal cells suggests that the antigen is exclusively located in the OM.

"Enterobacterial Common Antigen - II. Studies on the Immunogenic Form," by P. Kiss, Max-Planck-Institut für Immunbiologie, Freiburg

The immunogenic form of ECA is found only in R mutants. Functional rfe and rfaL genes - also known to be involved in LPS biosynthesis - are required for production of this immunogen: The gene product of the former for the biosynthesis of the haptenic form of ECA and the latter for the translocation of the ECA hapten onto the appropriate R core.

Comparative methylation analysis of degraded polysaccharide of an immunogenic *E. coli* strain and its ECA mutant revealed that the degree of the R core substituted by ECA is small (<5%). Anti-ECA IgG coupled to ferritin was incubated with *E. coli* R mutants possessing either the immunogenic form, haptenic form, or no ECA. The label was evenly distributed on the outer membrane in both ECA-positive strains.

Whole cell bacterial vaccines are generally toxic and often provide considerably less than complete protection. It has been recently demonstrated that purified capsular polysaccharide from *Neisseria meningitidis* groups A and C provide excellent protection while producing few adverse reactions. This development raises the possibility that other purified cell components might also be effective immunogens. Since cell envelope materials have extensive contact with host immune systems, they should be considered prime candidates for this purpose. Several recent reports suggest that major OM protein in *Neisseria* have potential as immunogens. ECA, whose location in cell envelopes was discussed in poster sessions at this Symposium, may have significance in prophylaxis and serodiagnosis of enterobacterial infections and the increasingly significant nosocomial infections. It was, therefore, disappointing that the immunogenic properties of membrane components were not addressed. Inclusion of such materials would have resulted in a lengthened program and might have created other problems from the organizational point of view. However, it is hard to imagine anyone regretting spending an additional day in such a delightful city.

#### CONCLUSION

The organization of this Symposium was nothing less than outstanding. A large lecture theatre at the Naturwissenschaftliche Institut, equipped with four screens and four large blackboards, was used for all lecture sessions. Speakers had wireless microphones and could freely move around the stage. All investigators scheduled to address the Symposium did so, and with high quality, well-prepared presentations. Poster presentations were made in a large foyer just outside the theatre. Coffee was also served in this foyer during both morning and afternoon sessions, and lunch was available in a nearby building.